

Synthesis and spectral characterization of environmentally responsive fluorescent deoxycytidine analogs

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Abbreviations: Ac₂O, acetic anhydride; Boc, *tert*-butyloxycarbonyl; Boc₂O, Boc anhydride; CAN, cerium ammonium nitrate; CI, chemical ionization; DMAP, 4-*N,N*-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; DNA, deoxyribonucleic acid; EDTA, ethylenediamine tetraacetic acid disodium salt; EI, electron impact; ESI, electron spray ionization; Et₃N, triethylamine; EtOH, ethanol; FCC, flash column chromatography; FRET, fluorescence resonance energy transfer; HRMS, high resolution mass spectrometry; MeCN, acetonitrile; MeOH, methanol; NBS, *N*-bromosuccinimide; NMR, nuclear magnetic resonance; PNA, peptide nucleic acid; pC, pyrrolocytosine; pdC, pyrrolodeoxycytidine; PydC, 5-(pyren-1-yl)deoxycytidine; RNA, ribonucleic acid; RT, room temperature; TBAF, tetrabutylammonium fluoride; TBDMS, *tert*-butyldimethylsilyl; THF, tetrahydrofuran; TLC, thin layer chromatography; TMS, trimethylsilyl; UV-VIS, ultraviolet-visible.

Herein, we describe the synthesis and spectroscopic properties of five novel pyrrolodeoxycytidine analogs, and the related 5-(1-pyrenylethynyl)-2'-deoxycytidine analog; as well as fluorescence characterization of 5-(*p*-methoxyphenylethynyl)-2'-deoxyuridine. Within this series of compounds, rigidification of the structure from 6-phenylpyrrolodeoxycytidine to 5,6-benzopyrrolodeoxycytidine made remarkable improvement of the fluorescence quantum yield ($\Phi \sim 1$, EtOH) and substantially increased the Stokes shift. Exchange of the phenyl group of 6-phenylpyrrolodeoxycytidine for other heterocycles (benzofuryl or indolyl) produced an increase in the extinction coefficient at the excitation wavelength while preserving high quantum yields. The steady-state fluorescence response to the environment was determined by sensitivity of Stokes shift to solvent polarity. The effect of solvent polarity on fluorescence emission intensity was concurrently examined and showed that 5,6-benzopyrrolodeoxycytidine is highly sensitive to the presence of water. On the other hand, the previously synthesized 5-(*p*-methoxyphenylethynyl)-2'-deoxyuridine was found to be sensitive to solvent viscosity indicating molecular rotor behavior.

Introduction

The design and synthesis of novel fluorescent probes capable of incorporation into DNA has experienced tremendous growth within the last two decades.¹⁻⁵ Naturally occurring nucleobases are virtually non-fluorescent under ambient conditions⁶ and thus require chemical modification to be endowed with desirable fluorescence properties. Such fluorescent properties may come from attachment of a pendent fluorophore (e.g. pyrene, anthracene).² Well-established synthetic protocols based on transition metal catalyzed cross-coupling reactions⁷ are oftentimes used for this purpose. Unfortunately, the introduction of a fluorophore extrinsic to the nucleobase often results in constructs possessing fluorescence properties insensitive to the change in the microenvironment of the nucleobase itself.¹⁻³ Taking this into

consideration, the development of fluorescent nucleobase analogs that function as reporters of microenvironmental change is of great interest.

An elegant solution to the problem outlined above would be the incorporation of fluorescent properties into the core of the nucleobase itself.¹⁻³ Such structural modifications should afford nucleobase analogs capable of reporting duplex formation or mismatch discrimination, preferably via changes in Stokes shift or fluorescence intensity. The term intrinsic fluorophores¹ has been coined to describe this group of nucleobase analogs. One goal of the design of intrinsically fluorescent nucleobase analogs is to make only modest structural changes when compared with their natural counterparts.¹ Another crucial design feature is to retain the Watson-Crick hydrogen bonding face so that canonical base pairing is not disturbed. When both of these

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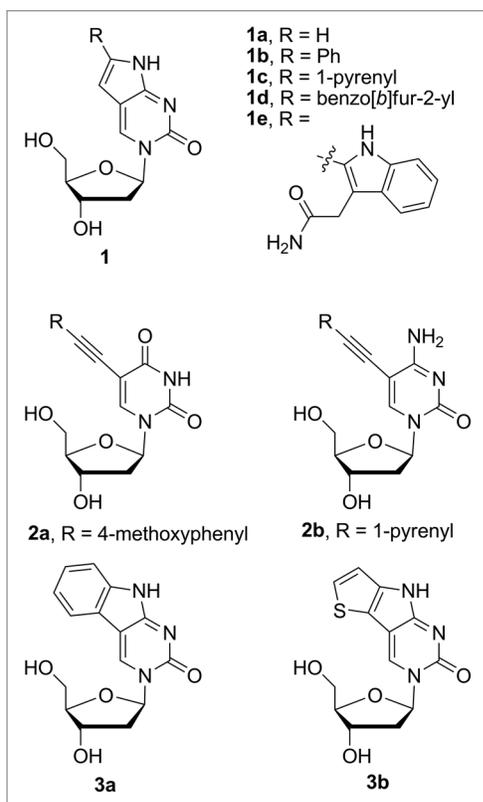


Figure 1. Nucleoside analogs **1a–e**, **2a**, **2b**, **3a**, and **3b**.

design criteria are met, the analog is referred to as an isomorphous nucleobase analog.² Such analogs are useful for probing nucleic acid structures, dynamics and interactions.² Despite the ever growing number of fluorescent nucleobase/nucleoside analogs reported, successful de novo design of nucleobase fluorophores is still very challenging and many analogs are produced by the familiar design-synthesize-evaluate (and repeat) paradigm.

As a result of a long standing interest in the development of intrinsically fluorescent nucleobases, we have investigated structural modifications of deoxycytidine and deoxyuridine.¹ It was found that the modest fluorescence associated with deoxypyrrolocytidine moiety^{8–11} (Fig. 1, **1a**) may be dramatically improved upon substitution of the position 6 of the parent heterocycle **1** with various substituted phenyl groups.^{12,13} Among them 6-phenylpyrrolocytidine¹⁴ (Fig. 1, **1b**) and some of its derivatives have been particularly well studied.^{15,16} These modified nucleobases were found to stabilize the DNA (RNA)-PNA duplexes¹⁶ and the fluorescence associated with them was found to be responsive to match duplex formation.¹⁵

To further investigate the effect of substitution at position 6 on the fluorescence properties of pyrrolocytidine, we have prepared several analogs including the incorporation of the large hydrocarbon-based fluorophore pyrene (Fig. 1, **1c**) and the heteroaromatics benzo[*b*]furan (Fig. 1, **1d**) and indole (Fig. 1, **1e**). Benzo[*b*]furan-modified nucleobases were shown to possess interesting fluorescence properties,¹⁷ while tryptophan residues within proteins and other indole derivatives are known to exhibit strong solvatochromism.^{18–20} It is also worth noting, that another

heteroaromatic 6-pyrrolocytosine nucleobase analog modified with thiophene (Fig. 1, **1f**) has been recently prepared and studied in Tor's laboratory.²¹

In order to investigate the role of the rotatable biaryl bond at the site of substitution on the fluorescence properties, we have prepared the 5,6-benzo-fused pyrrolocytidine analog (Fig. 1, **3a**) to compare with the properties of **1b**. A related thienyl-fused nucleobase analog (Fig. 1, **3b**) has recently been reported by Tor.²¹ Much earlier, Matteucci and von Krosigk synthesized **3a**, via a different synthetic route; however, its fluorescent properties were not reported.²²

In related studies, we have prepared the uncyclized C5-substituted 1-pyrenylethynyl-2'-deoxycytidine analog **2b** to compare with the pyrrolocytidine analog **1c** (Fig. 1). This analog has been prepared previously by semi-automated synthetic strategy using on-resin Sonogashira- cross-coupling,²³ but has not been characterized as the nucleoside. We have also included new characterization of the 4-methoxyphenylethynyl-substituted deoxyuridine analog **2a** (Fig. 1) which has been investigated previously and found to possess useful fluorescence properties as a hybridization probe.²⁴

Overall, we report the synthesis and spectral characterization of five pyrrolocytidine deoxynucleosides and a C5-ethynyl derivative of deoxycytidine (**2b**) and of deoxyuridine (**2a**). In particular, we have examined the effects of solvent polarity²⁵ and solvent viscosity²⁶ on the fluorescence associated these analogs.

Results and Discussion

Chemistry

The nucleoside analogs **1b–e** (Fig. 1) were obtained via a one-pot reaction cascade involving a Sonogashira cross-coupling between an appropriately hydroxyl-protected 5-iodo-2'-deoxycytidine and terminal alkyne followed by an intramolecular 5-endo-dig cyclization.¹² As previously reported, when modified reaction conditions were used, the products of the Sonogashira cross-coupling alone (Fig. 1, **2a** and **b**) were produced in high yields.¹³

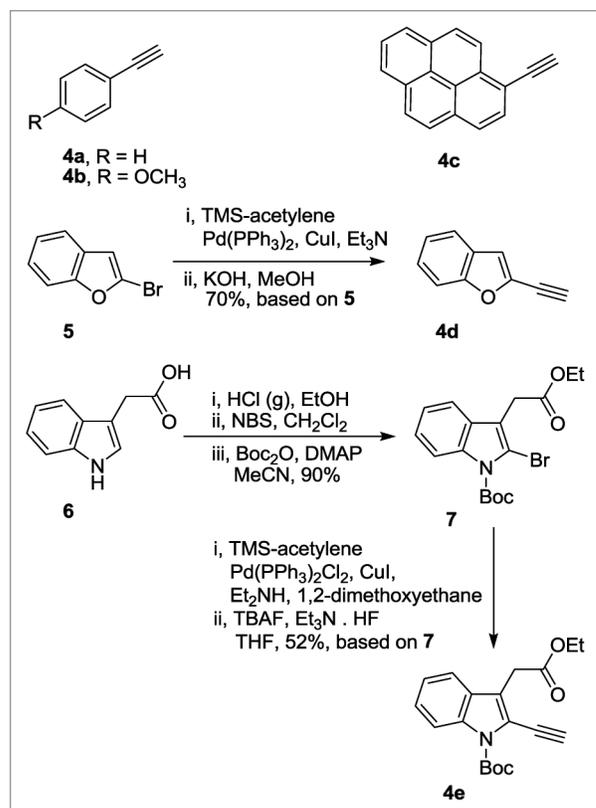
The structures and preparation of the required alkynes are shown in Scheme 1. Phenylacetylene (**4a**), 4-methoxyphenylacetylene (**4b**), and 1-ethynylpyrene (**4c**) were commercially available, while 2-ethynylbenzo[*b*]furan (**4d**) and ethyl 2-ethynyl-1-Boc-indole-3-yl acetate (**4e**) were synthesized. A slight modification of the recently described protocol²⁷ starting with Sonogashira cross-coupling of 2-bromobenzo[*b*]furan (**5**) with trimethylsilylacetylene (TMS-acetylene), followed by the removal of the TMS protecting group under basic conditions furnished the alkyne **4d** in 70% overall yield (Scheme 1). Indole-3-acetic acid (**6**) was esterified,²⁸ followed by the bromination at the 2 position²⁹ and Boc-protection of indole nitrogen. Ethyl 1-Boc-2-bromoindole-3-yl acetate (Scheme 1, **7**) was obtained in 90% yield, based on ethyl 2-bromoindole-3-yl acetate. Subsequent Sonogashira cross-coupling with TMS-acetylene followed by the removal of TMS protecting group³⁰ afforded the desired alkyne **4e** in 52% based on **7** (Scheme 1). Procedures describing the

preparation of alkynes **4d** and **4e** can be found in Materials and Methods section.

With alkynes **4a–e** in hand, the protected 5-iododeoxycytidine was subjected to Sonogashira cross-coupling (Scheme 2). The products of cyclization were obtained in serviceable yields (50–75%, based on **8a–c**) after purification by flash column chromatography (FCC). The acetyl groups in **1b–d** were removed in good yields (>75%) by treatment with K_2CO_3 in alcoholic solvents (MeOH or EtOH), and the products were purified once again by FCC. To obtain the desired indole-modified pyrrolo-cytidine analog **1e**, the product of cyclization was first treated with saturated solution of NH_3 in EtOH to effect amidation of the ethyl ester, followed by the removal of protecting *tert*-butyldimethylsilyl (TBDMS) groups by treatment with $Et_3N \cdot 3HF$ in THF thus providing **1e** in 68% yield (Scheme 2) after FCC purification.

When 5-iododeoxycytidine^{31,32} (**8a**) and 1-ethynylpyrene (**4c**) were subjected to the conditions of Sonogashira cross-coupling, the deoxycytidine analog **2b** was obtained in 50% yield (Scheme 2). The 4-methoxyphenyl-modified deoxyuridine analog **2a** was obtained in the same manner from deoxyuridine (**9**) and 4-methoxyphenylacetylene (**4b**) as described previously by our group²⁴ (Scheme 3).

The synthesis of 5,6-benzo-fused cytidine analog **3a** was performed similarly as for the related PNA monomer.³³ Deoxyuridine (**9**) was protected by acetylation of the hydroxyl groups, followed by bromination using a mixture of LiBr and cerium ammonium nitrate (CAN).³⁴ Subsequent reaction of the crude intermediate with $POCl_3$ and 1,2,4-triazole³³ afforded the deoxycytidine analog **10** (Scheme 3) in 51% overall based on **9**, after chromatographic purification. With intermediate **10** in hand, substitution at C4 with 2-bromoaniline was performed in wet dioxane. Although this reaction is well known, and usually performed under anhydrous conditions, we found that without some small amount of water present the reaction did not proceed well. After installation of the N4 group, the exocyclic nitrogen was masked with a Boc group³³ which provided the cyclization substrate **11** (49% overall yield, based on **10**, Scheme 3). The key step in the synthesis of the 5,6-benzo-fused cytosine analog **3a** was the reductive Ni-mediated cyclization,³⁵ performed in the same manner as described recently.³³ The protected 5,6-benzo-fused cytosine analog was obtained in 58% yield after chromatographic purification. It is interesting to point out, that the current synthetic yield represents *ca.* 2-fold improvement, compared with the synthesis of related 5,6-benzo-fused cytosine PNA monomer.³³ The removal of acetyl groups was effected by treatment with NH_4OH in MeOH, affording the desired monomer **3a** in 75% yield. The synthesis of the heterocyclic skeleton (pyrimido[4,5-*b*]indole) present in **3a** has been achieved previously in 6 steps and 15% overall yield starting from 5-iodo-deoxyuridine.²² Stille coupling of an appropriately substituted organotin moiety containing aniline with 5-iododeoxyuridine and subsequent DBU-mediated cyclization of the resulting heterobiaryl intermediate were the key steps in the previously described synthesis.²² The current synthesis of **3a** provided the desired product in 11% overall yield over 7 steps. Although proceeding in a marginally



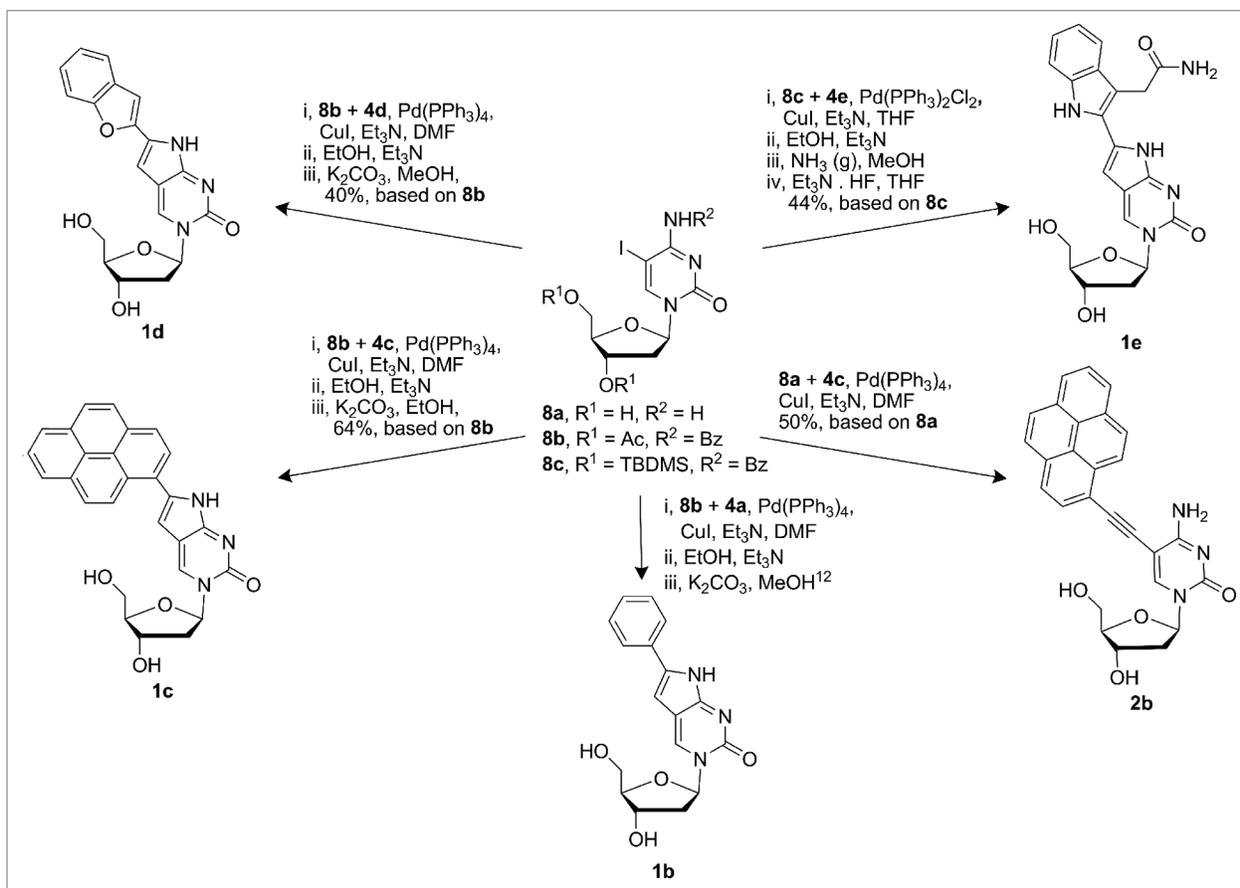
Scheme 1. Chemical structures and syntheses of alkynes **4a–e**.

lower yield, our synthesis has the advantages of avoiding toxic organotin reagents and only involves one protection/deprotection step whereas two are involved in the Matteucci synthesis.²² Moreover, intermediates **10** and **11** can also be utilized to prepare the nucleosides bearing various unusual heterocyclic skeletons, as described recently.³³

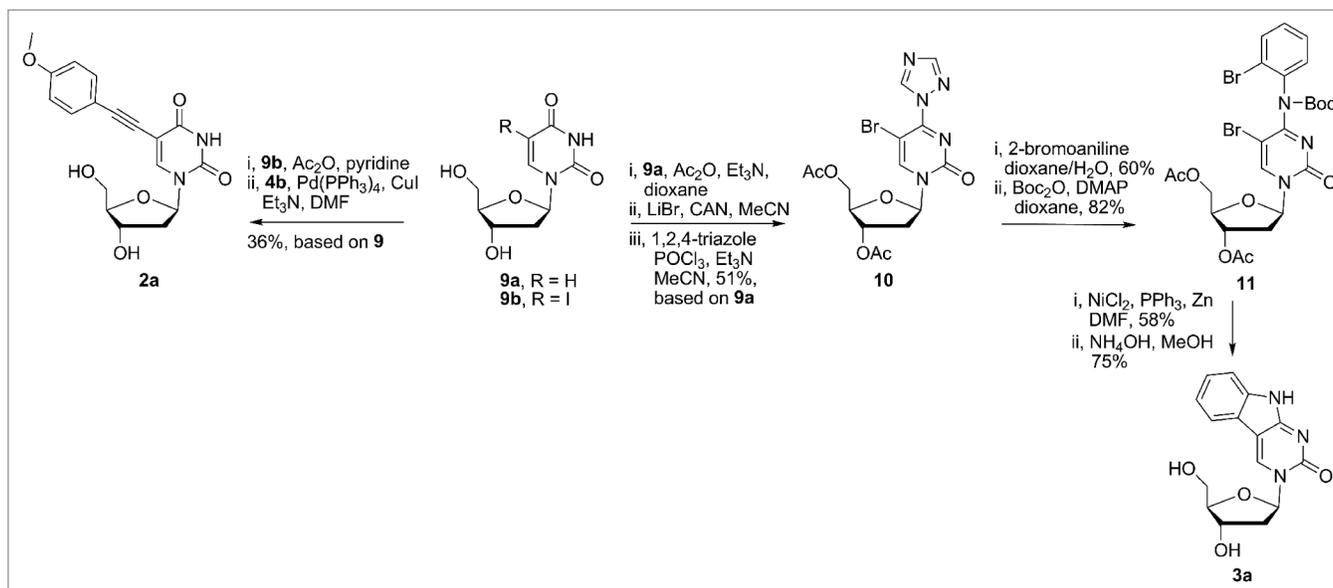
Fluorescence properties

The spectral properties of compounds **1b–e**, **2a**, **2b**, and **3a** have been studied in detail and are reported in Table 1. UV-Vis and fluorescence spectra were measured in EtOH, dioxane and water, in order to evaluate the influence of the polarity of the medium. Likewise, fluorescence quantum yields (Φ_f) and molar extinction coefficients (ϵ) have been determined for all of the compounds in each solvent, except for the molar extinction coefficients of **1c** and **2b** in pure water due to insufficient solubility.

In general, with comparison to the parent cytidine analog **1b** previously studied in our laboratory,¹² substitution at position 6 of pyrrolo-deoxycytidine with larger aromatic groups has a positive effect on both the quantum yield and extinction coefficients in organic solvents. Whereas the quantum yield of 6-phenylpyrrolo-deoxycytidine is good in organic solvent ($\Phi_f = 0.28$, dioxane), its ϵ value at the longest wavelength absorption band used for excitation is relatively small. The increased conjugation provided by the larger aromatic substituents makes a greater impact on the ϵ value and correspondingly leads to greater fluorophore brightness (defined as: $\epsilon \times \Phi_f$). Groups larger than phenyl (i.e., pyrenyl, benzo[*b*]furanyl, indolyl) also produce a bathochromic shift of



Scheme 2. Syntheses of nucleobase analogs **1b–e** and **2b**.



Scheme 3. Synthesis of the nucleobase analogs **2a** and **3a**.

the lowest energy absorption band. However, the absorption shift is relatively small (≤ 20 nm in all solvents examined) and results in the fluorophore excitation wavelength being in the near UV

and the emission being in the visible blue region. All of the pyrrolylcytidine analogs showed a response to the nature of the solvent with greater Stokes shift for the more polar solvent (H₂O) vs.

Table 1. Fluorescence properties of nucleobase analogs **1b-1e**, **2a**, **2b**, and **3a**

Compound	Structure	Solvent	λ_{ex}^a	λ_{em}^a	ϵ^b (10^3)	Φ_f	Stokes Shift ^a	Brightness ($\epsilon \times \Phi_f$) 10^3	Polarity Sensitivity ^c
1b		dioxane	376	441	3.5	0.28	65	1.0	
		EtOH	369	447	3.5	0.40	78	1.4	62
		water	362	454	2.4	0.31	92	0.7	
1c		dioxane	396	457	17.3	0.78	61	13.5	
		EtOH	388	443	16.0	0.60	55	9.6	34
		water	377	485	-	0.02	108	-	
1d		dioxane	382	445	15.4	0.44	63	6.8	
		EtOH	377	445	15.8	0.58	68	9.2	72
		water	372	462	13.5	0.50	90	6.8	
1e		dioxane	388	454	14.4	0.32	66	4.6	
		EtOH	388	472	10.4	0.48	84	5.0	93
		water	380	489	11.8	< 0.01	109	< 0.1	
2a		dioxane	334	412	19.9	0.18	78	3.6	
		EtOH	332	425	18.6	0.53	93	9.9	166
		water	353	463	17.9	0.06	110	1.0	
2b		dioxane	366	407	31.3	0.88	41	27.5	
		EtOH	363	393	31.0	0.55	30	17.0	-31
		water	383	394	-	< 0.01	11	-	
3a		dioxane	322	416	4.2	0.93	94	3.9	
		EtOH	322	433	6.8	> 0.99	111	6.8	105
		water	321	468	6.8	< 0.01	147	< 0.1	

^anm; ^b $\times 10^3 \text{ M}^{-1}\text{cm}^{-1}$; ^c $\text{cm}^{-1}/(\text{kcal} \cdot \text{mol}^{-1})$. pC = 6-substituted 2'-deoxypyrrroloctidine. dC = 5-substituted 2'-deoxycytidine, dU = 5-substituted 2'-deoxyuridine, dR = 2-deoxyribose.

ethanol or dioxane. The intensity of emission varied for the pyrroloctidine analogs within the dioxane, ethanol, and water series examined, but not in a uniform manner. Whereas 6-phenylpyrroloctidine **1b** showed near constant quantum yield in dioxane and water, the benzo[b]furanyl analog **1d** displayed an increased quantum yield while the 1-pyrenyl (**1c**) and indolyl (**1e**) exhibited dramatic quenching (Table 1). Although it was known that incorporation of **1b** into oligonucleotides yield constructs that maintain fluorescence in aqueous media,^{14,36} these initial results indicate that nucleoside analog **1d** may behave in a similar manner, yet be more emissive. In order to examine the effect of

the medium polarity on fluorescence emission, further studies were undertaken, *vide infra* (see "Solvent polarity effects").

The 6-pyrenyl, benzo[b]furanyl, and indolyl pyrroloctidine analogs are new compounds and, due to the lack of comparators, will be examined in relation to similar 5-substituted cytidine compounds. Firstly, 5-(pyren-1-yl)deoxycytidine (Pyc) has been prepared by Wagenknecht and used to study electron transport in DNA.^{37,38} Pyc demonstrated steady-state fluorescence in acetonitrile ($\lambda_{\text{excit.}} = 340 \text{ nm}$) with emission maximum at approximately 380 nm and showing some vibronic structure. It was posited that this emission resulted from a locally excited state, i.e. on the pyrene moiety. In water, the fluorescence was

dramatically quenched and this was ascribed to fast electron transfer to cytosine nucleobase followed by rapid protonation of the base radical. The analogous pyrenylpyrrolodeoxycytidine (**1c**) possesses a solvent dependent red-shifted absorbance ($\lambda_{\text{excit.}} = 377 - 396 \text{ nm}$) and emission ($\lambda_{\text{emiss.}} = 457 - 485 \text{ nm}$) with a markedly larger Stokes shift and dramatic quenching in water vs. ethanol or dioxane. The reduction potential of pyrroloctidine is not known; however, these results suggest that it is the electron acceptor from a photo-excited pyrene. Thusly, the large Stokes shift in water, nearly twice the magnitude as observed in ethanol or dioxane, argues for an excited state species with much greater polar character (or charge separated species) than the ground state. As well, the dramatic fluorescence quenching can be understood if the excited state was rapidly protonated, as for cytidine.

Next, 5-benzo[b]furanlycytidine has recently been reported as a member of the complete ribonucleoside family of benzo[b]furan conjugates.³⁹ Interestingly, this analog possessed rather large Stokes shifts in water, dioxane, methanol and acetonitrile; when excited at 320 nm and it displayed a broad emission centered near 450 nm for each solvent. This analog produced rather weak fluorescence ($\Phi_f < 0.06$) in a variety of solvents except for glycerol where the quantum yield was improved to 0.127. The pyrrolodeoxycytidine analog **1d** offers much improved fluorescence. In the three directly comparable solvents: dioxane, ethanol/methanol and water, **1d** possesses quantum yields > 0.4 and a red-shifted absorbance ($\lambda \sim 375 \text{ nm}$).

Finally, 5-indolylcytidine is unknown but a 5-tryptophanlycytidine derivative has been prepared by the photochemical reaction of 5-bromo-2'-deoxycytidine with *N*-acetyltryptophan ethyl amide.⁴⁰ While it was noted that this derivative was fluorescent in water ($\lambda_{\text{excit.}} = 350 \text{ nm}$; $\lambda_{\text{emiss.}} = 445 \text{ nm}$), the quantum yield was not reported. The pyrroloctidine analog **1e** exhibits a bathochromic shift relative to the aforementioned analog ($\lambda_{\text{excit.}} = 380 \text{ nm}$; $\lambda_{\text{emiss.}} = 489 \text{ nm}$) likely as a result of the pyrroloctidine nucleus vs. the cytosine. Compound **1e** shows fluorescence which is strongly dependent on the solvent. It has a high Φ_f in dioxane and ethanol, 0.32 and 0.48 respectively, while dramatic quenching (occurs in water ($\Phi < 0.01$)). The quenching may be a consequence of excited state electron transfer from the indole moiety to the base, similar to the proposed mechanism for the photoreaction reported,⁴⁰ followed by rapid protonation in analogy to the pyrene-conjugated nucleoside.

Rigidification of the heterocyclic skeleton present in analogs **1b–1e** to eliminate the possibility of free rotation about the biaryl bond produces analog **3a**. This strategy, *i.e.* fusion of rings to produce polycyclic heteroaromatic analogs of cytidine, has been previously employed for the preparation of fluorescent nucleosides. Notably, Matteucci's examples of phenothiazine and phenoxazine derivatives⁴¹ which were later described as highly emissive and environmentally insensitive fluorophores useful for fluorescence resonance energy transfer (FRET) based studies.^{42,43} Saito's group has reported fluorescently responsive cytidine analogs based on the pyridopyrimidine scaffold which can discriminate between the different environments presented by an opposing nucleoside in the context of hybridized oligonucleotides and has potential use in single-nucleotide polymorphism detection.^{44,45}

A structurally related example has been reported by Sekine for which geometrically locking a 4-*N*-carbamoyldeoxycytidine transformed the nonfluorescent precursor into a fluorescent analog that experienced quenching when paired to guanosine, but not adenosine, in the context of oligomers.⁴⁶

The proceeding examples are either bright but relatively insensitive to their environment or environmentally responsive but with low quantum yields (≤ 0.1) or small Stokes shift and peak emission in the UV which are not ideal properties. In the present case, nucleoside **3a** possesses remarkably high fluorescence quantum yields and large Stokes shift in the organic solvents of EtOH ($\Phi_f > 0.99$) and dioxane ($\Phi_f = 0.93$), while near complete quenching of the fluorescence is observed in water ($\Phi_f < 0.01$, Table 1). An increase in brightness factor is also observed originating from a larger ϵ value and greater Φ_f when compared with **1b**. These properties distinguish **3a** from known examples except for the structurally similar thiophene-fused analog recently reported by Tor;²¹ however, **3a** shows greater polarity sensitivity, as described below (see "Solvent polarity effects").

The non-cyclized 5-pyrenylethynyldeoxycytidine analog **2b** has been previously prepared via cross-coupling to a 5-iodocytidine already incorporated into an oligonucleotide but not as the free nucleoside.⁴⁷ Spectral characterization of a duplex containing a single 5-pyrenylethynyldeoxycytidine insert was described as pyrene-like exhibiting vibronic structure in the emission ($\lambda_{\text{emiss.}} = 408, 430 \text{ nm}$). It was concluded that there was weak electronic coupling between the pyrene moiety and cytosine base. Similarly, we note that the 5-pyrenylethynyldeoxycytidine analog **2b** has much different fluorescence spectra than pyrenylpyrrolodeoxycytidine **1c** (see Fig. S2–S4). Whereas the qualities of the spectra of **1c** resemble other pdC analogs, *i.e.*, a red-shifted absorption relative to the precursor components – in this case cytosine and ethynylpyrene, and a broad, featureless emission $> 450 \text{ nm}$; the spectra of **2b** is distinctly different retaining vibronic structure and lacking a large bathochromic shift. In our description of nucleobase fluorophores, **1c** behaves as an integrated fluorophore and **2b** behaves more like a pendant fluorophore.

Solvent polarity effects

Nucleoside probes that respond fluorimetrically to polarity find use in the interrogation of nucleic acid structures. Such responsive nucleosides can be deliberately designed, such as the 6-dimethylamino-2-acylnaphthalene-deoxyuridine conjugate synthesized by Saito and coworkers exploiting the known polarity responsiveness of the substituted naphthalene moiety.^{48,49} Alternatively, the property of polarity sensitivity may be discovered serendipitously when the primary goal is endowing a nucleoside with fluorescence. During the past few years, Tor and coworkers have established methods to characterize the polarity dependent fluorescence of nucleoside analogs. Recently, they have described the use of a simple binary solvent system (dioxane and water in varying proportions) in order to adjust the polarity of the medium while minimizing changes in other parameters, such as solvent viscosity.²¹ The variation in Stokes shift is then plotted against Reichardt's microscopic solvent polarity parameter to quantify the polarity sensitivity.⁵⁰ In order to make direct

comparisons with previously reported data, we have adopted the Tor method to evaluate the change in fluorescence with the respect to changes in medium polarity. The variation of Stokes shift and emission intensity as the solvent polarity was changed was determined for each of the nucleoside analogs **1b–1e**, **2a**, **2b**, and **3a** and is summarized in Table 1 while the spectral data are presented in Figures 2 and 3 and the Supplemental Materials.

From the Stokes shifts dependence on the microenvironmental polarity parameter, the parent nucleoside analog **1b** (Fig. 2A) displays moderate polarity sensitivity ($62 \text{ cm}^{-1}/[\text{kcal} \cdot \text{mol}^{-1}]$, Table 1) and is comparable to that associated with the benzofuranyl-containing analog **1d** (Fig. 2C). Nucleoside **1d** not only possesses a much improved quantum yield over the previously reported 5-benzofuranylcytidine, the polarity sensitivity is also much larger $72 \text{ cm}^{-1}/(\text{kcal} \cdot \text{mol}^{-1})$ vs. $-26 \text{ cm}^{-1}/(\text{kcal} \cdot \text{mol}^{-1})$ estimated from data presented by Srivatsan.³⁹

The indole-modified analog **1e** possesses greater polarity sensitivity [$93 \text{ cm}^{-1}/(\text{kcal} \cdot \text{mol}^{-1})$] likely reflecting the properties of the indole group (Fig. 2D). The variation of tryptophan fluorescence with the environment is a known feature exploited in studying the protein conformation.^{18,19}

The Stokes shifts of the pyrene-containing derivatives **1c** (Fig. 2B) and **2b** (Fig. 2E) are less sensitive to changes in the solvent polarity (Table 1), and show no evidence of aggregation or excimer formation. The lack of Stokes shift response to polarity change is not unexpected based on the well known insensitivity of pyrene itself. However, it is notable that the pyrenylpyrrolocytosine derivative **1c** exhibits a weak positive effect consonant with the other pyrrolocytosine derivatives, and different than the pyrenylethynyl-dC **2b**, which also supports the notion that **1c** is an integrated fluorophore with substantial electronic communication between the pyrene and pC moieties.

The 5,6-benzo-fused analog **3a** ($105 \text{ cm}^{-1}/[\text{kcal} \cdot \text{mol}^{-1}]$, Table 1, Fig. 2F) possesses greater sensitivity of Stokes shift to solvent polarity than the unfused congener **1b**. Interestingly, **3a** is extremely sensitive to the presence of water; the addition of 10% water results in ca. 94% reduction in fluorescence intensity. This behavior is obviously different than the overall trend observed for analogs **1b–1e**, **2a**, and **2b** which display a non-unidirectional effect on emission intensity with the increasing polarity, providing the maximum fluorescence emission in dioxane solutions containing ca. 10–40% of water. The extraordinary sensitivity to the presence of water may be useful property for examining nucleoside binding proteins/transporters or after the incorporation into oligonucleotides to report on ligand/protein/enzyme binding that excludes access of water to the fluorophore.

Solvent viscosity effects

It has been well documented that fluorescent nucleosides that feature a rotatable bond as part of the chromophore can display an increase of fluorescence intensity in the solvents with increased viscosity.²⁶ With the exception of analog **3a**, all analogs described in this study possess a potentially rotatable C-C biaryl (or alkyne-aryl) bond as part of the fluorophore. We have, therefore also decided to investigate the relationship between the emission intensity and viscosity of the solvents.

Our studies began with examining the emission intensity of parent analog 6-phenylpyrroloxydeoxycytidine **1b** in mixtures of MeOH and glycerol that vary in viscosity. The emission intensity associated with **1b** was found to be independent of solvent viscosity; therefore none of remaining pyrrolocytidine analogs **1c–1e** were studied. However, it was observed previously that the phenylethynyldeoxyuridine **2a** exhibited strong “turn on” of fluorescence in a duplex vs. single-stranded state. It was suspected that **2a** may exhibit molecular rotor properties based on this observation of increased fluorescence in a more restricted environment (duplex) than one that would permit free rotation about the alkyne-aryl bonds (single strand). The nucleoside **2a** has been evaluated as shown in Figure 3.

The phenylethynyldeoxyuridine **2a** displays a substantial response to changes in solvent polarity (Fig. 3, top panel), possessing the highest value yet reported (measured under comparable conditions) for neutrally-charged nucleosides.^{21,51} It also displays a trend of increased fluorescence in solvents mixtures of higher viscosity, at the near constant polarity of methanol/glycerol solvent mixtures (Fig. 3, bottom panel), which indicates molecular rotor behavior.²⁶

In contrast, to the properties of **2a**, the pyrenylethynyldeoxycytidine **2b** showed no clear dependence on solvent viscosity (data not shown). This behavior can be understood in the following way. The phenylethynyluracil moiety is entirely part of the fluorophore and the rotatable bonds are integral to the fluorophore structure whereas the pyrene moiety is the fluorophore and the rotatable bonds are external to it, thus having little effect on the fluorescence. This notion is supported by the observation that the fluorescence emission of **2b** shows vibronic structure not dissimilar to pyrene (Fig. S3) and is very similar to that reported for 1-phenylethynylpyrene.⁵² In addition, the lack of response of the Stokes shift to changes in solvent polarity parallels pyrene itself which shows approximately constant emission wavelength but changes in relative intensities of the so-called I_1 and I_3 bands which form the basis of the *Py* scale.⁵³

Materials and Methods

General remarks

Reagents were commercially available unless otherwise stated and all solvents were reagent grade except for water (18.2 MΩ·cm millipore water). Dry solvents (dioxane, DMF) for chemical synthesis were obtained by drying on activated Al_2O_3 columns in a solvent purification system. Spectroscopic grade solvents (dioxane, DMSO, glycerol, EtOH, MeOH) have been used to perform spectroscopic studies. Solvents were removed under reduced pressure in a rotary evaporator and organic extracts were dried over Na_2SO_4 . Reaction mixtures involving air sensitive reagents ($\text{Pd}[\text{PPh}_3]_4$, CuI) have been degassed by passing the stream of N_2 gas into the reaction mixture cooled to $-78 \text{ }^\circ\text{C}$. The flask was then evacuated and the passing of N_2 was repeated twice more. FCC was performed using silica gel (SiO_2 ; mesh size 230–400 Å). Thin-layer chromatography (TLC) was performed on an Al

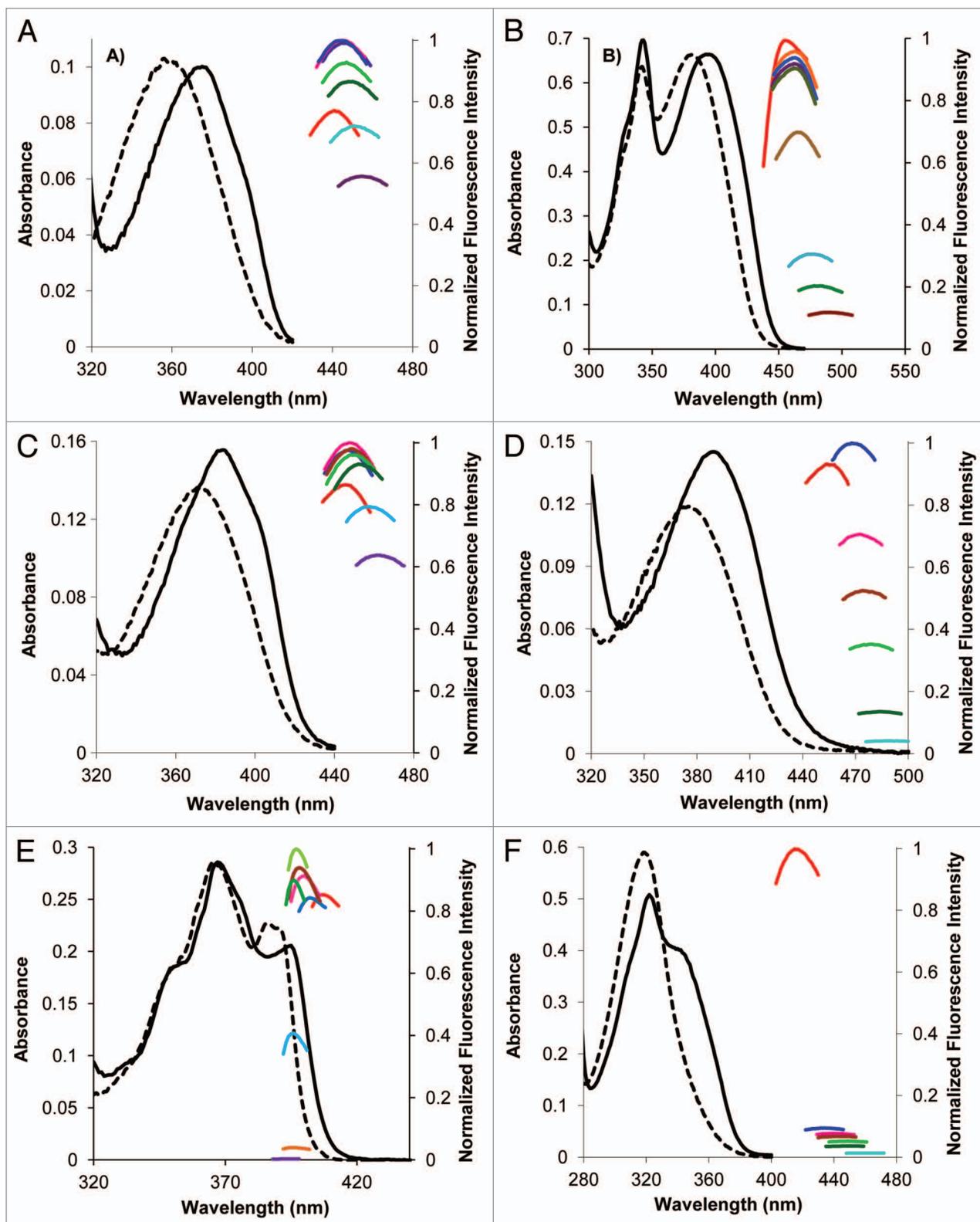


Figure 2. Photophysical characterization of cytidine analogs. Absorbance in water (dashed black line) and dioxane (solid black line) and emission (100% dioxane, red; 90% dioxane/10% water, blue; 80% dioxane/20% water, pink; 70% dioxane/30% water, brown; 60% dioxane/40% water, light green; 40% dioxane/60% water, dark green; 20% dioxane/80% water, turquoise; 10% dioxane/90% water, orange; 100% water, purple). (A) **1b**; (B) **1c**; (C) **1d**; (D) **1e**; (E) **2b**; (F) **3a**.

backed silica gel plate with compounds visualized by I₂ vapors, 5% ninhydrin stain, phosphomolybdic acid stain, and UV light. ¹H and ¹³C NMR spectra were recorded on a 400 MHz spectrometer. Chemical shifts (δ) are reported in parts per million, and are referenced as follows: CDCl₃ (7.26 ppm), DMSO-D₆ (2.49 ppm) for ¹H NMR and CDCl₃ (77.0 ppm), DMSO-D₆ (39.5 ppm) for ¹³C NMR (100 MHz). Mass spectra (MS) were obtained on a mass spectrometer using electron impact (EI), chemical ionisation (CI), or electrospray ionisation (ESI). UV-VIS spectra were acquired using an UV-VIS spectrophotometer. Steady-state fluorescence spectra were acquired using a fluorimeter. All fluorescence measurements were performed using a 1 cm wide four-sided quartz cuvette. UV-VIS measurements were performed using a 1 cm two-sided quartz glass cuvette.

Synthesis of 2-ethynylbenzo[*b*]furan (4d)

2-Bromobenzo[*b*]furan (5591 mg, 3 mmol) was dissolved in Et₃N (10 mL), followed by the addition of Pd(PPh₃)₂Cl₂ (42 mg, 0.06 mmol) and CuI (6 mg, 0.03 mmol). The mixture was degassed followed by the addition of TMS-acetylene (640 μL, 4.5 mmol). The mixture was then stirred (in the atmosphere of N₂) for 2 h at 60 °C, was cooled to room temperature (RT), the solid residue formed during the reaction was filtered off with suction and the filter was washed with hexanes. The filtrate was concentrated; the residue was dissolved in MeOH (6 mL) containing KOH (168 mg, 3 mmol). The mixture was stirred for 30 min at RT, the solvent was evaporated and the residue was subjected to FCC on 50 g SiO₂ eluted with light petroleum ether. The eluate was concentrated to afford 2-ethynylbenzo[*b*]furan (4d, 300 mg, 70%, based on 5) as colorless oil. The spectral data for 4d were in agreement with those previously reported.²⁷

Synthesis of indole containing alkyne 4e

Ethyl 1-Boc-2-bromoindole-3-yl acetate (7) was prepared from 2-bromoindole-3-yl acetic acid (6) according to literature procedures.^{28,29} Subsequently the alkyne functionality was installed in two steps. Ethyl 1-Boc-2-bromoindole-3-yl acetate (7, 3.8 g, 10 mmol) and TMS-acetylene (4.1 mL, 29.5 mmol) were mixed with diethylamine (6 mL) and dimethoxyethane (6 mL). The mixture was degassed followed by the addition of Pd(PPh₃)₂Cl₂ (350 mg, 0.5 mmol) and CuI (300 mg, 1.5 mmol). The reaction mixture was then stirred (in the atmosphere of N₂) for 12 h at 55 °C. The solvent was evaporated and the residue was subjected to FCC on 100 g SiO₂ eluted with hexanes/EtOAc (98:2) to afford ethyl 1-Boc-2-(2-TMS-ethynyl)indole-3-yl acetate (2.6 g, 65%). ¹H NMR (CDCl₃): δ = 7.92 (d, 1H, *J* = 8.6), 7.23 (d, 1H, *J* = 7.8), 7.07 (t, 1H, *J* = 7.8), 6.96 (m, 1H), 3.87 (q, 2H, *J* = 7.0), 3.54 (s, 2H), 1.40 (s, 9H), 0.97 (t, 3H, *J* = 7.0), 0.0 (s, 9H). ¹³C NMR (CDCl₃): δ = 169.8, 149.0, 135.5, 128.1, 125.8, 122.9, 122.7, 118.9, 115.4, 104.4, 95.8, 84.1, 60.6, 31.0, 27.9, 13.96, -0.33. HRMS (EI): calcd. for C₂₂H₂₉NO₄Si [M⁺] 399.1866, found 399.1873.

Ethyl 1-Boc-2-(2-TMS-ethynyl)indole-3-yl acetate (1.6 g, 4 mmol) was dissolved in THF (6 mL) and the solution was cooled to -10 °C. Et₃N · HF (0.6g, 16 mmol) was added to the stirred mixture followed by a dropwise addition (over the period of 15 min) of 1 M solution of TBAF in THF (8 mL, 8 mmol). The

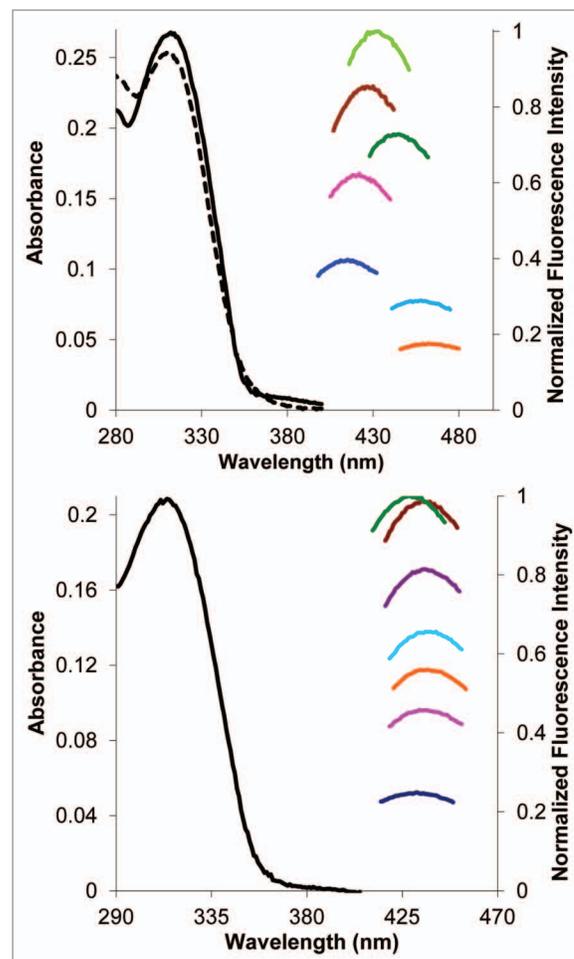


Figure 3. Photophysical characterization of uridine analog **2a**. Top panel: fluorescence changes in response to changes in polarity. Absorbance in water (dashed black line) and dioxane (solid black line) and emission in 9:1 dioxane/water, blue; 8:2 dioxane/water, pink; 7:3 dioxane/water, brown; 6:4 dioxane/water, light green; 4:6 dioxane/water, dark green; 2:8 dioxane/water, turquoise; 1:9 dioxane/water, orange. Bottom panel: fluorescence response to changes in medium viscosity, absorbance in MeOH (solid black line) and emission in MeOH, blue; 7:3 MeOH/glycerol, pink; 1:1 MeOH/glycerol, orange; 4:6 MeOH/glycerol, turquoise; 2:8 MeOH/glycerol, purple; 15:85 MeOH/glycerol, brown; 1:9 MeOH/glycerol, green.

mixture was allowed to warm up to 0 °C and was stirred for 1 h at 0 °C. The precipitate formed during the reaction was filtered off using a short plug of SiO₂, the filter was washed with hexanes/EtOAc (1:1) and the filtrate was concentrated. The residue was then subjected to FCC on 50 g SiO₂, hexanes/EtOAc (98:2) to afford ethyl 1-Boc-2-ethynylindole-3-yl acetate (**4e**, 1.06 g, 80%). ¹H NMR (CDCl₃): δ = 8.12 (d, 1H, *J* = 7.8), 7.49 (d, 1H, *J* = 7.4), 7.33 (t, 1H, *J* = 7.4), 7.23 (t, 1H, *J* = 8.0), 4.12 (q, 2H, *J* = 7.0), 3.83 (s, 2H), 3.64 (s, 1H), 1.66 (s, 9H), 1.20 (t, 3H, *J* = 7.0). ¹³C NMR (CDCl₃): δ = 169.6, 148.8, 135.2, 127.8, 125.6, 127.7, 122.4, 118.9, 118.0, 115.2, 86.7, 84.1, 74.6, 60.5, 30.6, 27.6, 13.7. HRMS (EI): calcd. for C₁₉H₂₁NO₄ [M⁺] 327.1471, found 327.1479.

Spectral data associated with all of the intermediates were in agreement with those published previously,³⁰ see supplemental information.

Synthesis of 4-benzoyl-5'-iodo-5',3'-di-Ac-deoxycytidine (8b)

Deoxycytidine (1.06 g, 4.67 mmol) was dissolved in AcOH (10 mL, sonication required), followed by the addition of CHCl₃ (10 mL) and acetyl chloride (4 mL). The mixture was then stirred for 18 h at RT; was cooled to 0 °C and excess acetyl chloride was quenched by the addition of MeOH (4 mL). The solvents were evaporated, the residue was coevaporated with toluene (ca. 100 mL), followed by dissolving the residue in saturated NaHCO₃ solution (50 mL). The product was extracted with 8 × 50 mL of CH₂Cl₂/MeOH (95:5), combined organic extract was dried and was concentrated to leave 5',3'-di-*O*-Ac-deoxycytidine (1.44 g, 99%) as colorless oil of sufficient purity to be used in the next step. ¹H NMR (DMSO-*d*₆): δ = 9.58 (br,s, 1H), 8.62 (br,s,1H), 7.92 (d, *J*₁ = 7.82 Hz, 1H), 6.16 (m, 1H), 6.09 (t, *J*₁ = 6.84 Hz, 1H), 5.18 (m, 1H), 4.23 (br,s,3H), 2.41 (m, 2H), 2.06 (s, 3H), 2.04 (s, 3H). ¹³C NMR (DMSO-*d*₆): δ = 170.19, 170.06, 160.66, 148.30, 143.78, 94.47, 86.11, 81.88, 73.83, 63.57, 36.58, 20.78, 20.63. HRMS (ESI) *m/z* calcd. for C₁₃H₁₈N₃O₆ [MH⁺] 312.1196, found 312.1198.

Iodine (698 mg, 2.75 mmol) and iodic acid (418 mg, 2.38 mmol) were added to a vigorously stirred mixture containing 5',3'-di-*O*-Ac-deoxycytidine (1.44 g, 4.63 mmol) in AcOH (10.5 mL), CCl₄ (7 mL) and water (7 mL). Resulting mixture was stirred vigorously for 24 h at 40 °C, the volatiles were evaporated and the residue was partitioned between saturated NaHCO₃ solution (50 mL) and EtOAc (50 + 2 × 30 mL). Combined organic extract was dried, was concentrated and was subjected to FCC on 60 g SiO₂ eluted with CH₂Cl₂/MeOH (98:2) later replaced with CH₂Cl₂/MeOH (95:5). Evaporation of the eluate afforded 5-iodo-5',3'-di-*O*-Ac-deoxycytidine (1.12 g, 55%) as pale yellow oil. ¹H NMR (DMSO-*d*₆): δ = 9.14 (br, s, 1H), 8.47 (br, s, 1H), 8.17 (s, 1H), 6.06 (t, *J*₁ = 6.84 Hz, 1H), 5.18 (m, 1H), 4.26 (m, 3H), 2.47 (m, 1H), 2.37 (m, 1H), 2.09 (s, 1H), 2.06 (s, 1H). ¹³C NMR (DMSO-*d*₆): δ = 170.06, 170.01, 161.34, 149.97, 148.52, 86.18, 81.91, 73.81, 63.51, 57.31, 48.61, 36.76, 20.78. LRMS (EI) *m/z* calcd. for C₁₃H₁₆IN₃O₆ [M⁺] 437.0084, found 437.0084.

Benzoic anhydride (748 mg, 3.31 mmol) was added to a solution of 5-iodo-5',3'-di-*O*-Ac-deoxycytidine (1.11 g, 2.54 mmol) in dry pyridine (8 mL). The mixture was stirred for 3 h at 85 °C (N₂ atmosphere). Pyridine was removed by coevaporation with toluene (3 × 100 mL), the residue was then partitioned between saturated aq. NaHCO₃ (50 mL) and EtOAc (50 + 30 mL). Combined organic extract was dried, was concentrated and was subjected to FCC on 40 g SiO₂ eluted with hexanes/acetone (2:1). The eluate was concentrated to ca. one quarter of its original volume (crystallization of the product was visible) and was set aside for 1 h at -10 °C. Separated crystals were filtered off, were washed with hexanes and were dried to leave *N*4-benzoyl-5'-iodo-5',3'-di-*O*-Ac-deoxycytidine **8b** (883 mg, 64%) as colorless crystals. ¹H NMR (DMSO-*D*₆): δ 12.86 (s, D₂O exch., 1H); 8.22 (m, 3H); 7.63 (m, 1H); 7.52 (m, 2H); 6.12 (t, *J* = 6.5 Hz, 1H); 5.21 (m, 1H); 4.28 (m, 3H); 2.54 (m, 1H); 2.39 (m, 1H); 2.13

(s, 3H); 2.07 (s, 3H). ¹³C NMR (DMSO-*D*₆): δ 178.1, 170.1, 170.0, 156.2, 147.2, 146.5, 136.2, 132.9, 129.5, 128.4, 86.0, 82.0, 73.8, 70.0, 63.5, 36.5, 20.8 (2 × C). HRMS (ESI) *m/z*: found 542.0404 [M+H]⁺ (542.0424 calcd. for C₂₀H₂₁IN₃O₇).

Synthesis of 4-benzoyl-5'-iodo-5',3'-di-TBDMS-deoxycytidine (8c)

Benzoic anhydride (4.1 g, 18 mmol) was added to a suspension of crude 5'-iodo-5',3'-di-*O*-TBDMS-deoxycytidine (prepared from 5.5 mmol of 5'-iododeoxycytidine according to the literature procedure⁴⁴) in MeCN (25 mL) followed by the addition of DMAP (20 mg, 0.17 mmol). Resulting mixture was stirred (atmosphere of N₂) for 8 h at 70 °C. The solvent was evaporated; the residue was subjected to FCC on 100 g SiO₂, eluted with CH₂Cl₂/MeOH (98:2) later replaced with CH₂Cl₂/MeOH (95:5) to afford *N*4-benzoyl-5'-iodo-5',3'-di-*O*-TBDMS-deoxycytidine (**8c**, 3.2 g, 85%). ¹H NMR (CDCl₃): δ 13.25 (s, D₂O exch., 1H); 8.38 (m, 2H); 8.25 (s, 1 H); 7.55 (m, 1H); 7.47 (d, 2 H); 6.29 (dd, *J* = 8.0, 5.5 Hz, 1H); 4.41 (m, 1H); 4.03 (dd, *J* = 4.5, 2.0 Hz, 1H); 3.93 (dd, *J* = 11.5, 2.5 Hz, 1H); 3.79 (dd, *J* = 11.5, 2.5 Hz, 1H), 2.39 (ddd, *J* = 13.0, 8.0, 2.0 Hz, 1H); 2.02 (ddd, *J* = 13.5, 8.0, 6.0 Hz, 1H); 0.96 (s, 9H); 0.90 (s, 9H); 0.18 (s, 3H); 0.17 (s, 3H); 0.10 (s, 3H); 0.09 (s, 3H). ¹³C NMR (CDCl₃): δ 179.7, 156.9, 147.4, 145.6, 136.6, 132.8, 130.2, 128.2, 88.7, 86.4, 72.6, 69.3, 63.0, 42.2, 26.2, 25.7, 18.5, 18.0, -4.7, -4.9, -5.1, -5.3. HRMS (ESI) *m/z*: found 708.1786 [M+Na]⁺ (708.1762 calcd. for C₂₈H₄₄IN₃O₅Si₂Na).

General protocol for the tandem Sonogashira cross-coupling/5-endo-dig cyclization cascade

Compound **1b** was prepared as described in the literature.¹² A round bottom flask containing *N*4-benzoyl-5-iodo-5',3'-di-*O*-Ac-deoxycytidine (**8b**, 420 mg, 0.78 mmol) and corresponding alkynes as follows: 2-ethynylpyrene (**4c**, 265 mg, 1.17 mmol); 2-ethynylbenzo[*b*]furan (**4d**, 197 mg, 1.39 mmol) and ethyl 1-Boc-2-ethynylindole-3-yl acetate (**4e**, 457 mg, 1.4 mmol) was charged with N₂ and dry DMF (3 mL, alkynes **4c** and **4d**) or dry THF (2.5 mL, alkyne **4e**) was added. The mixtures were degassed followed by the addition of Pd(PPh₃)₄ (alkynes **4c** and **4d**, 90 mg, 0.08 mmol) or Pd(PPh₃)₂Cl₂ (alkyne **4e**, 56 mg, 0.08 mmol) and CuI (30 mg, 0.16 mmol). The mixtures were degassed again, Et₃N (1.08 mL, 7.76 mmol) was added and the mixtures were stirred (in the dark, under N₂ atmosphere) for 18 h at 50 °C (alkyne **4c**), for 8 h at rt and 18 h at 60 °C (alkyne **4d**) and for 24 h at 50 °C (alkyne **4e**). EtOH and Et₃N were added (2 mL each) and the stirring continued for 18 h at 50 °C (alkyne **4c**), 18 h at 80 °C (alkyne **4d**) and for 18 h at 55 °C (alkyne **4e**). The mixtures were cooled to RT, were diluted with 4% EDTA solution (50 mL) and were extracted with CH₂Cl₂ (2 × 25 mL, alkyne **4c**), or EtOAc (2 × 30 + 20 mL, alkyne **4d**). Combined organic extracts were washed with brine (3 × 50 mL), were dried and were concentrated. The residues were subjected for FCC purification on 50 g SiO₂ eluted with toluene later replaced with toluene/MeOH (98:2, alkyne **4c**) or 30 g SiO₂ eluted with Et₂O/acetone (3:1) later replaced with Et₂O/acetone (2:1, alkyne **4d**). In the case of alkyne **4e** no aqueous extractive workup was performed, the solvent was evaporated and the residue was subjected for FCC on 60 g SiO₂ eluted with CH₂Cl₂/MeOH (98:2) later

replaced with CH₂Cl₂/MeOH (95:5). Evaporation of the eluates afforded the desired products derived from alkynes **4d** and **4e** of sufficient purity. In the case of product derived from the alkyne **4c**, the impurities were removed by adding the solution of the cyclization product in CH₂Cl₂ (ca. 2 mL) into hexanes (ca. 20 mL). The precipitate was separated by filtration, washed with hexanes and was dried to afford 6-(2-pyrenyl)-pyrrolo-5',3'-di-*O*-Ac-deoxycytosine (296 mg, 75%) as yellow solid. ¹H NMR (DMSO-D₆): δ 12.02 (s, D₂O exch., 1H); 8.62 (s, 1H); 8.52 (m, 1H); 8.36 (m, 3H); 8.25 (m, 4H); 8.13 (m, 1H); 6.74 (s, 1H); 6.38 (t, *J* = 6.5 Hz, 1H); 5.27 (d, *J* = 6.5 Hz, 1H), 4.37 (m, 3H), 2.62 (m, 1H), 2.42 (m, 1H), 2.11 (s, 3H), 2.09 (s, 3H). ¹³C NMR (DMSO-D₆): δ 170.3, 170.1, 159.9, 153.9, 138.8, 136.5, 130.9 (2 × C), 130.4, 128.3, 128.1, 128.0, 127.3, 127.1, 126.6, 126.3, 125.8, 125.4, 124.9, 124.3, 124.2, 123.8, 109.7, 102.0, 87.4, 82.1, 74.2, 63.8, 38.0, 20.8, 20.7. HRMS (ESI) *m/z* found 536.1816 [M+H]⁺ (calcd. 536.1822 for C₃₁H₂₆N₃O₆).

6-(2-Benzo[*b*]furan-2-yl)-pyrrolo-5',3'-di-*O*-Ac-deoxycytosine (186 mg, 53%), yellow solid. ¹H NMR (DMSO-D₆): δ 11.87 (s, D₂O exch., 1H); 8.57 (s, 1H); 7.71 (d, *J* = 8 Hz, 1H); 7.63 (d, *J* = 8 Hz, 1H); 7.35 (m, 2H); 7.29 (dd, *J* = 8, 8 Hz, 1H); 6.79 (s, 1H); 6.29 (t, *J* = 6.5 Hz, 1H); 5.23 (m, 1H); 4.34 (m, 3H), 2.73 (m, 1H); 2.38 (m, 1H); 2.09 (s, 3H); 2.06 (s, 3H). HRMS (ESI) *m/z*: found 452.1468 [M + H]⁺ (calcd. 452.1458 for C₂₃H₂₂N₃O₇).

Ethyl 6-[(1-Boc-indole-3-yl acetate)-2-yl]-pyrrolo-5',3'-di-*O*-TBDMS-deoxycytosine (394 mg, 64%), yellow solid. ¹H NMR (CDCl₃): δ 9.42 (br s, 1 H); 8.77 (s, 1H); 8.21 (d, *J* = 8.0, 1H); 7.58 (d, *J* = 8.0, 1H); 7.39 (dd, *J* = 8.0, 8.0, 1H); 7.29 (dd, *J* = 8.0, 8.0, 1H); 6.42 (s, 1H); 6.39 (m, 1H); 4.41 (dd, *J* = 11.5, 6.0, 1H); 4.17 (q, *J* = 7.5, 2H); 4.03 (m, 1H); 4.00 (m, 1H); 3.85 (m, 1H); 3.68 (s, 2H); 2.61 (m, 1H); 2.21 (m, 1H); 1.42 (s, 9H); 1.26 (t, *J* = 7.0, 1H); 0.95 (s, 9H); 0.89 (s, 9H); 0.17 (s, 3H); 0.14 (s, 3H); 0.07 (2 × s, 6H). ¹³C NMR (CDCl₃): δ 171.1, 158.5, 154.5, 149.5, 136.6, 136.3, 131.0, 128.8, 127.9, 125.7, 123.1, 119.3, 117.1, 115.7, 108.6, 102.4, 87.7, 84.2, 69.8, 61.7, 61.3, 42.6, 31.2, 27.8, 26.0, 25.7, 18.4, 17.9, 14.2, -4.5, -5.0, -5.4 (2 × C). HRMS (ESI) *m/z*: found 803.3860 [M+Na]⁺ (calcd. 803.3847 for C₄₀H₆₀N₄O₈Si₂Na).

Removal of the protecting groups from the cyclization products derived from alkynes **4c** and **4d**

Separate suspensions of 6-(1-pyrenyl)pyrrolo-5',3'-di-*O*-Ac-deoxycytosine (100 mg, 0.19 mmol) and K₂CO₃ (5 mg, 0.04 mmol) in EtOH (20 mL) and 6-(benzo[*b*]fur-2-yl)pyrrolo-5',3'-di-*O*-Ac-deoxycytosine (205 mg, 0.45 mmol) and K₂CO₃ (157 mg, 1.13 mmol) in MeOH (6 mL) were stirred at RT (to form **1c**) or at 0 °C (to form **1d**), until the TLC analysis revealed the complete consumption of starting material. The solids were removed by filtration, the filtrates were evaporated and the residues were purified by trituration (CH₂Cl₂, ca. 10 mL) and subsequent crystallization using EtOH/water (product **1c**) or by FCC on 20 g SiO₂ eluted with EtOAc/MeOH (95:5) later replaced with EtOAc/MeOH (9:1), followed by the evaporation of the eluate.

6-(1-Pyrenyl)pyrrolo-deoxycytosine (**1c**, 84 mg, 85%) as yellow solid. ¹H NMR (DMSO-D₆): δ 11.97 (s, D₂O exch., 1H); 8.86 (s, 1H); 8.52 (d, 1H); 8.36 (m, 3H); 8.25 (m, 4H); 8.13 (m, 1H);

6.69 (s, 1H); 6.33 (t, *J* = 6.5 Hz, 1H); 5.32 (d, D₂O exch., *J* = 4.0 Hz, 1H); 5.17 (t, D₂O exch., *J* = 5.0, 1H); 4.30 (m, 1H); 3.94 (m, 1H); 3.69 (m, 2H), 2.42 (ddd, *J* = 13.5, 6.0, 4.0 Hz, 1H), 2.09 (ddd, *J* = 13.5, 6.0, 4.0 Hz). ¹³C NMR (DMSO-D₆): δ 159.6, 154.0, 138.9, 136.9, 130.9, 130.8, 130.4, 128.3, 128.1, 128.0, 127.3, 127.1, 126.6, 126.4, 125.8, 125.4, 124.9, 124.2, 124.1, 123.9, 109.3, 101.9, 88.0, 87.2, 70.1, 61.1, 41.6. HRMS (ESI) *m/z* found 452.1601 [M+H]⁺ (calcd. 452.1610 for C₂₇H₂₂N₃O₄).

6-(Benzo[*b*]furan-2-yl)pyrrolo-deoxycytidine (**1d**, 124 mg, 75%) as yellow solid. ¹H NMR (DMSO-D₆): δ 12.08 (s, D₂O exch., 1H); 8.79 (s, 1H); 7.70 (d, *J* = 8 Hz, 1H); 7.62 (d, *J* = 8 Hz, 1H); 7.36 (m, 2H); 7.28 (dd, *J* = 8, 8 Hz, 1H); 6.73 (s, 1H); 6.24 (t, *J* = 6.5 Hz, 1H); 5.30 (d, D₂O exch., *J* = 4.5 Hz, 1H); 5.15 (t, D₂O exch., *J* = 5.5 Hz, 1H); 4.25 (m, 1H); 3.92 (m, 1H), 3.67 (m, 2H), 2.39 (m, 1H); 2.05 (m, 1H). ¹³C NMR (DMSO-D₆): δ 159.7, 154.3, 153.8, 148.0, 137.6, 130.0, 128.2, 125.3, 123.6, 121.5, 111.1, 108.6, 103.5, 98.6, 88.0, 87.2, 69.9, 61.0, 41.5. HRMS (ESI) *m/z*: found 368.1228 [M + H]⁺ (calcd. 368.1246 for C₁₉H₁₈N₃O₅).

Removal of the protecting groups from the cyclization product derived from alkyne **4e**

Ethyl 6-2-[(1-Boc-indole-3-yl acetate)-2-yl]-pyrrolo-5',3'-di-*O*-TBDMS-deoxycytosine (250 mg, 0.32 mmol) was added to a solution of NH₄OH (ca. 28%, 1 mL) in MeOH (4 mL) cooled to 0 °C in a sealed vessel. The mixture was then stirred for 12 h at 55 °C, the solvent was evaporated and the residue was purified by FCC on 30 g SiO₂ eluting with CH₂Cl₂/MeOH (98:2) later replaced with CH₂Cl₂/MeOH (95:5). The eluate was evaporated and the residue was dissolved in THF (3 mL), the solution was cooled to 0 °C, followed by the addition of Et₃N · 3HF (155 mg, 1 mmol). The mixture was then stirred at RT for 12 h, the solvent was evaporated, the residue was triturated with Et₂O (ca. 20 mL), the product was crystallized from MeOH to afford 6-[(indole-3-yl acetamide)-2-yl]pyrrolo-deoxycytosine (**1e**, 92 mg, 68%), yellow solid. ¹H NMR (DMSO-D₆): δ 12.47 (s, D₂O exch., 1H); 11.37 (s, D₂O exch., 1H); 8.77 (s, 1H); 8.06 (s, D₂O exch., 1H); 7.82 (m, 1H); 7.45 (s, D₂O exch., 1H); 7.37 (m, 1H); 7.15 (m, 1 H); 7.05 (m, 1 H); 6.59 (s, 1H), 6.27 (t, *J* = 6.0 Hz, 1H); 5.26 (m, D₂O exch., 1H); 5.14 (m, D₂O exch., 1H); 4.26 (m, 1H); 3.90 (m, 1H); 3.66 (m, 4H); 2.37 (m, 1H); 2.05 (m, 1H). ¹³C NMR (DMSO-D₆): δ 174.5, 159.2, 153.8, 136.4, 136.0, 132.4, 128.4, 127.7, 122.6, 119.3, 118.9, 111.1, 109.1, 107.5, 97.8, 87.9, 87.0, 69.7, 64.9, 60.9, 41.5. HRMS (ESI) *m/z*: found 424.1622 [M + H]⁺ (calcd. 424.1621 for C₂₁H₂₂N₃O₅).

Sonogashira cross-coupling to form the analogs **2a** and **2b**

Uridine analog **2a** was prepared from **4b** and **9b** according to the literature procedure; spectral data were consistent with those reported previously.²⁴

5-Iododeoxycytidine (**8a**, 500 mg, 1.41 mmol) and 1-ethynylpyrene (**4c**, 410 mg, 1.83 mmol) were dissolved in dry DMF (5 mL). The mixture was degassed, then Pd(PPh₃)₄ (160 mg, 0.14 mmol) and CuI (53 mg, 0.28 mmol) were added and the mixture was degassed again. Et₃N (720 μL, 5.64 mmol) was then added and the mixture was stirred (in the dark, under N₂ atmosphere) for 18 h at RT. The solvent was evaporated; the residue was subjected to FCC on 30 g SiO₂ eluting with CH₂Cl₂/MeOH

(95:5). Evaporation of the eluate afforded 5-(1-pyrenylethyn-2-yl)deoxycytidine (**2b**, 310 min, 50%), yellow solid. ¹H NMR (DMSO-*D*₆): δ 8.63 (s, 1H); 8.58 (m, 1H); 8.43 (m, 1H); 8.34 (m, 4H); 8.23 (m, 2H); 8.13 (m, 1H); 7.92 (s, D₂O exch., 1H); 7.26 (s, D₂O exch., 1H); 6.19 (t, *J* = 6.0 Hz, 1H); 5.28 (m, D₂O exch., 2H); 4.30 (m, 1H); 3.86 (m, 1H); 3.74 (m, 1H); 3.67 (m, 1H); 2.24 (m, 1H); 2.15 (m, 1H). ¹³C NMR (DMSO-*D*₆): δ 163.8, 153.4, 145.4, 130.8, 130.7, 130.6, 130.5, 129.8, 128.6, 128.2, 127.2, 126.7, 125.9, 125.8, 125.2, 124.7, 123.6, 123.4, 117.1, 92.6, 57.6, 87.5, 85.6, 69.7, 60.8, 56.0, 40.9. HRMS (ESI) *m/z* found 452.1590 [M+H]⁺ (calcd. 452.1610 for C₂₇H₂₂N₃O₄).

Preparation of 4-[(1,2,4-triazole)-1-yl]-5-bromo-5',3'-di-Ac-deoxycytidine

Ac₂O (4 mL, 42 mmol) was added to a solution of deoxyuridine (**9a**, 1.14 g, 5 mmol) and Et₃N (4 mL, 29 mmol) in dry dioxane (15 mL). The mixture was stirred for 24 h at RT and then the solvent was evaporated. The residue was taken up into water (25 mL) and extracted with EtOAc (3 × 40 mL). The combined organic extracts were dried and then concentrated to afford the desired 5',3'-di-*O*-Ac-deoxyuridine (pale yellow oil) of sufficient purity for the subsequent step. 5',3'-Di-*O*-Ac-deoxyuridine (1.56 g, 5 mmol), LiBr (521 mg, 6 mmol) and CAN (5.48 g, 10 mmol) were suspended in MeCN (70 mL) and stirred for 90 min at 80 °C. The solvent was evaporated, the residue was taken up into brine (50 mL) followed by the extraction with EtOAc (4 × 50 mL). The combined organic extract was dried and concentrated to afford 5-bromo-5',3'-di-*O*-Ac-deoxyuridine (pale yellow oil) of sufficient purity for the subsequent step. 1,2,4-Triazole (3.11 g, 45 mmol) was dissolved in MeCN (190 mL) and the resulting solution was cooled to 0 °C. Et₃N (10.5 mL, 75 mmol) was added, followed by the dropwise addition of POCl₃ (1 mL, 11 mmol). The mixture was stirred for 30 min at 0 °C at which point a solution of 5-bromo-5',3'-di-*O*-Ac-deoxyuridine (2.11 g, 5 mmol) in MeCN (10 mL) was added dropwise over a period of ca. 10 min. The cooling bath was removed and the mixture was heated at 60 °C for 18 h. The solvent was removed by evaporation, the residue was taken up into brine (50 mL), followed by the extraction with EtOAc (4 × 50 mL). The combined organic extract was dried and concentrated. The residue was subjected to FCC on 75 g SiO₂ eluting with CH₂Cl₂/acetone (4:1). Evaporation of the eluate afforded 4-[(1,2,4-triazole)-1-yl]-5-bromo-5',3'-di-*O*-Ac-deoxycytidine (**10**, 1.13 g, 51%, based on **9a**), pale yellow oil. ¹H NMR (CDCl₃): δ 9.16 (s, 1H); 8.53 (s, 1H); 8.18 (s, 1H); 6.25 (t, *J* = 6.5 Hz, 1H); 5.24 (m, 1H); 4.45 (m, 1H); 4.43 (m, 2H); 2.93 (ddd, *J* = 15.0, 6.5, 3.0 Hz, 1H); 2.23 (m, 1H); 2.16 (s, 3H); 2.13 (s, 3H). ¹³C NMR (CDCl₃): δ 170.3, 170.0, 155.8, 153.5, 152.1, 148.7, 145.2, 88.2, 86.8, 83.7, 73.4, 63.3, 39.2, 20.9, 20.8. HRMS (ESI) *m/z*: found 442.0366 [M+H]⁺ (442.0362 calcd. for C₁₅H₁₇BrN₃O₆).

Preparation of 4-Boc, 4-(2-bromophenyl)-5-bromo-5',3'-di-Ac-deoxycytidine (**11**)

To install the *N*₄-aryl group, 2-bromoaniline (447 mg, 2.6 mmol) and (1,2,4-triazol-1-yl)-5-bromo-5',3'-di-*O*-Ac-deoxycytidine (**10**, 884 mg, 2 mmol) were dissolved in dioxane (5.4 mL) containing water (600 μL). The mixture was stirred for 48 h at RT. The solvent was evaporated; the residue was taken

up into water (40 mL) and was extracted with EtOAc (2 × 50 mL). The combined organic extract was dried and was concentrated. The residue was subjected to FCC on 60 g SiO₂ eluting with CH₂Cl₂/acetone (93:7). Evaporation of the eluate afforded *N*₄-(2-bromophenyl)-5-bromo-5',3'-di-*O*-Ac-deoxycytidine (650 mg, 60%), white solid. ¹H NMR (CDCl₃): δ 8.74 (dd, *J* = 8.5, 1.5 Hz, 1H); 8.03 (s, D₂O exch., 1H); 8.00 (s, 1H), 7.58 (dd, *J* = 8.0, 1.5 Hz, 1H); 7.38 (m, 1H); 7.03 (m, 1H); 6.31 (dd, *J* = 7.5, 6.0 Hz, 1H); 5.23 (m, 1H); 4.39 (m, 2H); 4.34 (m, 1H), 2.76 (ddd, *J* = 14.5, 8.0, 6.0 Hz, 1H); 2.17 (s, 3H); 2.13 (m, 1H); 2.12 (s, 3H). ¹³C NMR (CDCl₃): δ 170.4, 170.2, 157.3, 153.7, 140.4, 135.1, 132.3, 128.5, 125.8, 123.1, 114.7, 89.0, 87.0, 82.8, 74.0, 63.7, 39.0, 20.9 (2 × C). HRMS (ESI) *m/z*: found 543.9705 [M+H]⁺ (543.9719 calcd. for C₁₉H₂₀Br₂N₃O₆).

To install the Boc protecting group, a solution of Boc₂O (786 mg, 3.6 mmol) in dry dioxane (6 mL) was added to a flask containing *N*₄-(2-bromophenyl)-5-bromo-5',3'-di-*O*-Ac-deoxycytidine (650 mg, 1.2 mmol). DMAP (15 mg, 0.12 mmol) was then added and the mixture was stirred at 50 °C for 18 h. The solvent was evaporated and the residue was subjected to FCC on 50 g SiO₂ eluted with CH₂Cl₂/acetone (19:1). Evaporation of the eluate afforded *N*₄-Boc, *N*₄-(2-bromophenyl)-5-bromo-5',3'-di-*O*-Ac-deoxycytidine (**11**, 636 mg, 82%), white solid. ¹H NMR (CDCl₃): δ 8.21 (s, 1H); 7.61 (m, 1H); 7.31 (m, 2H); 7.19 (m, 1H); 6.20 (dd, *J* = 7.0, 6.0 Hz, 1H); 5.22 (m, 1H); 4.38 (m, 3H); 2.81 (ddd, *J* = 14.5, 8.0, 2.5 Hz, 1H); 2.16 (s, 3H); 2.15 (m, 1H); 2.11 (s, 3H). ¹³C NMR (CDCl₃): δ 170.3, 170.2, 164.3, 153.5, 151.2, 143.9, 139.2, 133.4, 130.7, 129.4, 128.1, 95.7, 87.7, 83.8, 83.3, 73.8, 63.5, 39.1, 27.8, 20.9, 20.9 (2 × C). HRMS (ESI) *m/z*: found 644.0264 [M+H]⁺ (644.0243 calcd. for C₂₄H₂₈Br₂N₃O₈).

Ni-mediated cyclization and deprotection to form 5,6-benzofused analog **3a**

Zn dust (392 mg, 6 mmol), NiCl₂ (52 mg, 0.4 mmol) and PPh₃ (420 mg, 1.6 mmol) were placed in a flask and flushed with N₂ for 45 min. Dry DMF (2 mL) was added under a continuous flow of N₂ gas and the solution was heated to 80 °C until a dark red color persisted (ca. 20 min). A solution of *N*₄-Boc, *N*₄-(2-bromophenyl)-5-bromo-5',3'-di-*O*-Ac-deoxycytidine (**11**, 258 mg, 0.4 mmol) in dry DMF (2 mL) was added dropwise over a period of ca. 5 min. The reaction was then stirred for 1 h at 80 °C (N₂ atmosphere), cooled to RT, and then quenched with a 4% solution of EDTA (50 mL). The aqueous phase was extracted with EtOAc (4 × 50 mL), the combined organic extract was dried and then concentrated. The residue was subjected to FCC on 35 g SiO₂ eluted with CH₂Cl₂/MeOH (98:2) which was later replaced with CH₂Cl₂/MeOH (95:5) to afford the diacetylated precursor of the analog **3a** (90 mg, 58%), white solid. ¹H NMR (DMSO-*D*₆): δ 11.63 (s, D₂O exch., 1H); 8.83 (s, 1H); 7.87 (d, *J* = 8.0 Hz, 1H); 7.34 (ddd, *J* = 8.0, 8.0, 1.0 Hz, 1H); 7.27 (d, *J* = 8.0 Hz, 1H); 7.19 (ddd, *J* = 8.0, 8.0, 1.0 Hz, 1H); 6.33 (dd, *J* = 8.0, 6.0 Hz, 1H); 5.27 (m, 1H); 4.38 (m, 2H); 4.35 (m, 1H); 2.56 (ddd, *J* = 14.5, 8.5, 2.5 Hz, 1H); 2.42 (m, 1H); 2.10 (s, 3H); 2.04 (s, 3H). ¹³C NMR (DMSO-*D*₆): δ 170.2, 170.1, 161.8, 154.3, 140.1, 136.5, 126.8, 121.4, 120.2, 111.3, 109.5, 104.1, 87.2, 82.1, 74.3, 63.7, 37.6, 20.8, 20.6. HRMS (ESI) *m/z*: found 386.1336 [M+H]⁺ (386.1352 calcd. for C₁₉H₂₀N₃O₆).

The free nucleoside was accessed by: NH_4OH (ca. 28%, 4.5 mL) was added to a solution of the diacetylated precursor of the analog **3a** (90 mg, 0.23 mmol) in MeOH (4.5 mL) and the mixture was stirred for 2 h at 55 °C. Volatiles were evaporated and the desired analog **3a** (52 mg, 75%) was purified by crystallization from MeOH. $^1\text{H NMR}$ ($\text{DMSO-}D_6$): δ 11.64 (s, D_2O exch., 1H); 9.14 (s, 1H); 7.79 (d, $J = 8.0$ Hz, 1H); 7.32 (dd, $J = 8.0, 8.0$ Hz, 1H); 7.26 (d, $J = 8.0$ Hz, 1H); 7.17 (dd, $J = 8.0, 8.0$ Hz, 1H); 6.26 (t, $J = 6.0$ Hz, 1H); 5.29 (m, D_2O exch., 1H); 4.28 (m, 1H); 3.90 (m, 1H); 3.72 (m, D_2O exch., 2H); 2.35 (ddd, $J = 13.5, 10.5, 4.5$ Hz, 1H); 2.10 (m, 1H). $^{13}\text{C NMR}$ ($\text{DMSO-}D_6$): δ 161.6, 154.4, 140.0, 136.7, 126.5, 121.2, 120.6, 119.9, 111.3, 103.6, 87.7, 86.7, 69.5, 60.7, 41.3. HRMS (ESI) m/z : found 302.1119 $[\text{M}+\text{H}]^+$ (302.1141 calcd. for $\text{C}_{15}\text{H}_{16}\text{N}_3\text{O}_4$).

Quantum yields determination

Quantum yields (Φ) associated with compounds **1b–e**, **2a**, **2b**, and **3a** were determined in dioxane, EtOH and water. All quantum yields (Φ) measured in water were determined using quinine bisulfate in 0.5 M H_2SO_4 (Φ 0.55) as a standard.⁵⁴ Quantum yields (Φ) measured in organic solvents for **1c**, **1e**, and **2b** were determined using 9,10-dichloro-anthracene (Φ 0.55) as a standard,⁵⁵ for **2a** and **3a** pyrene (Φ 0.53) was used as a standard⁵⁵ and for **1b** and **1d** 9,10-diphenyl-anthracene (Φ 0.90) was used as a standard.⁵⁴ In each case the following equation was used to calculate the relative fluorescence quantum yield using seven samples of varying concentrations.

$$\Phi_s = \frac{I_s}{I_{ref}} \cdot \frac{Abs_{ref}}{Abs_s} \cdot \Phi_{ref}$$

Φ (Fluorescence quantum yield), I (integrated fluorescence intensity) and Abs (absorbance at λ_{ex}). The standard is denoted as (*ref*) while the sample is denoted as (*s*).

Molar extinction coefficients

Molar extinction coefficients (ϵ) associated with compounds **1b–e**, **2a**, **2b**, and **3a** were estimated by constructing Beer-Lambert plots ($A = \epsilon \times c \times l$), wherein A (absorbance), ϵ (molar extinction coefficient), c (concentration), l (light path length ~ 1 cm). Molar extinction coefficients (ϵ) associated with compounds **1b–e**, **2a**, **2b**, and **3a** were determined in dioxane, EtOH, and water with the exception of **1c** and **2b**, wherein an insufficient solubility prevented the determination of molar extinction coefficients (ϵ) in water.

Solvent polarity study

Stock solutions of compounds **1b–e**, **2a**, **2b**, and **3a** (on the order of 10^{-3} M) were prepared in DMSO. These stock solutions were diluted ($\sim 10\times$, depending on fluorophore brightness) in dioxane. These solutions were then further diluted ($25\times$) into volumetric flasks with appropriate volumes of water and dioxane to prepare working solutions containing 100%, 90%, 80%, 70%, 60%, 40%, 20%, and 10% dioxane in water. A 100% water sample was prepared in similar fashion using an equivalent amount of the DMSO solution. The $E_T(30)$ values were obtained from the literature.⁵⁶ Excitation and emission spectra for each sample were then obtained with the measurement of the maxima over a narrow window (approx. 50 nm, average of ten independent

measurements). Plots of solvent polarity ($E_T(30)$) vs. Stokes shift (cm^{-1}) were then constructed (see Supplemental Materials) and the slope was calculated to provide a numerical representation of solvent polarity sensitivity in $\text{cm}^{-1}/(\text{kcal mol}^{-1})$. This procedure is similar to the method reported by Tor and colleagues.²¹ Points were excluded if the fluorescence emission was too low to reliably find a maximum in either the excitation, or the emission spectra. The points obtained for 100% water and 100% dioxane solutions associated with compound **2a** were not taken into consideration due to excessive changes in the electronic structure between the neat and binary solutions preventing the selection of a constant electronic transition to monitor. For the compounds **1c** and **2b** a significant shift in the linear trend can be observed. As such the largest continuously linear segments were used to represent the polarity sensitivity of the compound.

Solvent viscosity study

Solutions of 70%, 50%, 40%, 20%, 15%, and 10% methanol in glycerol (5 mL each) were prepared by stirring the solvent mixtures overnight (RT) in sealed vials to ensure homogeneity. A known amount of compound **2a** was then dissolved in DMSO to obtain a stock solution with the concentration 2.79×10^{-3} M. Then, 20 μL of the stock solution was added to the solutions of glycerol in MeOH prepared previously. UV-Vis absorbance, excitation and emission spectra were then obtained with the emission maximum being averaged over a small window (50 nm). Integrated emission intensities were then normalized using Equation 1. The log of the normalized emission intensities were then plotted (Fig. 3) vs. the log of the solvent viscosity, obtained using equation (2).

$$I_{norm} = \frac{0.1}{Abs_{\epsilon_{ex}}} \cdot I \quad (1)$$

$$\ln \zeta_{mix} = \sum_{i=1}^2 w_i \cdot \ln \zeta_i \quad (2)$$

I (Integrated fluorescence intensity), Abs (absorbance at λ_{ex}), η (viscosity), and w (weight fraction). The normalized data are denoted as (*norm*) and the data for mixtures are denoted as (*mix*) respec. Values of 1317 cp (glycerol) and 0.583 cp (MeOH) have been used for the viscosity of the solvents.²⁶ Values of 1.25 kg/L (glycerol) and 0.79 kg/L (MeOH) have been used for the density of the solvents.

Conclusions

The syntheses of five novel pyrrolocytidine analogs (**1c–e**, **3a**) and a 5-ethynylcytidine (**2b**) analog have been achieved that usefully expand the repertoire of environmentally responsive fluorescent nucleosides. The photophysical characterization of the new analogs has been done along with previously reported nucleosides **1b** and **2a** for comparison.^{12,24}

The pyrrolocdeoxycytidine analogs **1c–e** display fluorophore behavior that is congruent with 6-phenylpyrrolodeoxycytidine **1b**, that is, they have the property of being an intrinsic

fluorophore, even for the large pyrene substituent. The similarity in fluorescence emission, a broad featureless band at $\lambda > 450$ nm, argues for substantial electronic communication between the pC nucleus and the 6-substituent. This is in contrast to the emission of the related 5-pyrenylethynyldeoxycytidine which resembles more closely an isolated pyrene, as previously noted.⁴⁷ The benefit to variation of the 6-substituent is shown by improvement of fluorophore brightness, primarily by increasing the extinction coefficient at the longest wavelength absorption band used for excitation. As well, variation of the nature of the heteroaromatic substituent at position 6 produces compounds with varying sensitivity to the polarity of the environment, as judged by the emission Stokes shift and emission intensity. For example the benzo[*b*]furan-2-yl-modified analog **1d** is approximately six times brighter than the parent analog **1b**, yet possesses similar polarity sensitivity and high emission intensity in water. The indole-containing analog **1e** displays not only greater polarity sensitivity, in terms of Stokes shift, but also shows a dramatic change in quantum yield and emission intensity in water. These limited examples tantalizingly suggest: (1) the pyrrolocytosine nucleus is a suitable scaffold for intrinsic nucleobase fluorophore development; and (2) that it may be possible to discover application specific analogs with a desirable combination of responsiveness to water, medium polarity and also possessing high brightness.

For 6-phenylpyrrolodeoxycytidine, the archetypal fluorophore possessing a biaryl bond, solvent viscosity showed no systematic effect on the emission intensity indicating that rotation about this bond is not a significant influence on the radiationless decay of the excited state. Similarly, 5-pyrenylethynyldeoxycytidine (**2b**) exhibited no dependence of the fluorescence intensity on medium viscosity; however, the spectral properties of **2b** indicate that the origin of this effect is different than for **1b**. Whereas **1b** exhibits spectral properties of an intrinsic nucleobase fluorophore wherein the nucleobase and the 6-substituent (phenyl) form an integrated fluorophore, the pyrene group in **2b** appears more independent of the nucleobase. Thus, the rotatable linkage

between the nucleobase and the pyrene is not within the core structure of the fluorophore and consequently has little influence on the radiationless relaxation of the excited state. For comparison, we turned to a fluorophore with an integrated acetylenic linkage in the core of the structure: 5-(*p*-methoxyphenylethynyl)deoxyuridine (**2a**). For this compound, a strong dependence on solvent viscosity was found resembling that observed for known molecular rotors.²⁶

Although **1b** did not show molecular rotor behavior, rigidification of pyrrolocytosine moiety (elimination of the rotatable C-C biaryl bond) to furnish the 5,6-benzo-fused analog **3a** produced a large positive impact on the quantum yield in organic solvents ($\Phi \sim 1.0$) and brightness (compared with parent **1b**). Further characterization of the 5,6-benzo-fused pyrrolocytidine analog **3a** showed that it is extraordinarily sensitive to the presence of water; combined with a high quantum yield and large Stokes shift, makes this a promising nucleoside probe. The sensitivity of fluorophore **3a** may be exploited in studies involving environments with strict exclusion of water, such as DNA-protein interactions.⁵⁷

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Copies of ¹H and ¹³C NMR spectra, steady-state fluorescence spectra in EtOH, dioxane, and water, data for determination of solvent polarity sensitivity and data for response to solvent viscosity for **2a** are available. Supplemental materials may be found here: www.landesbioscience.com/journals/artificialdna/article/29174

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